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Effects of Estrogen Deficiency and Bisphosphonate Therapy on Osteocyte Viability and
Microdamage Accumulation in an Ovine Model of Osteoporosis

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Running Title: Osteocyte Apoptosis and Microdamage

Abstract

It has been proposed that osteocyte viability plays an important role in bone integrity, and that bone loss in osteoporosis may be partially due to osteocyte cell death following estrogen depletion. Osteoporosis treatments such as bisphosphonates can inhibit osteocyte apoptosis which in turn may also reduce remodelling. Consequently, microcracks in bone which are normally repaired by bone remodelling may accumulate. This study used an ovine model of osteoporosis to examine the effects of estrogen depletion and bisphosphonates on osteocyte apoptosis and microdamage accumulation.

Skeletally mature ewes were randomly assigned into two equal groups; ovariectomy (OVX) and a non-treatment group (control). Half of these animals were sacrificed twelve months post-OVX. 20 months post-OVX, a number of OVX animals were randomly selected and each received a supra-pharmacological dose of the bisphosphonate, zoledronic acid (Zol). This group and all the remaining animals were sacrificed 31 months post-OVX. A compact bone specimen was removed from the left metacarpal of each animal; half was used for osteocyte apoptosis detection and the remainder for microdamage analysis.

Estrogen deficiency resulted in significant increases in the levels of osteocyte apoptosis while zoledronic acid significantly reduced the level of apoptosis in osteocytes. Zoledronic acid treatment resulted in the formation of more microcracks. However, these cracks were shorter than in control or OVX groups which may provide one explanation as to why increased damage levels following bisphosphonate treatment have not lead to increased fractures. This study also provides additional evidence of the importance of estrogen in preserving the osteocyte network.

Key Words: Osteoporosis, Ovine, Osteocyte, Apoptosis, Microcrack, Bisphosphonate

Introduction

Although there are a number of factors involved in determining the risk of developing osteoporosis, the end result is an imbalance between resorption by osteoclasts and formation by the osteoblasts. The consequence of which is a loss of bone resulting in fracture and for this reason most treatments for the disease focus primarily on preventing osteoclast activity. However, it has been suggested that osteocyte viability may also play a significant role in the maintenance and integrity of bone, and that bone loss in osteoporosis may be due in part to osteocyte cell death following estrogen depletion. ^(1,2) Previous studies have found that estrogens prevent the apoptosis of osteocytes. ⁽³⁻⁵⁾ Studies have also linked osteocyte apoptosis to the initiation of the bone remodelling process and that osteocyte density decreases with age. ^(6,7) All of these studies imply that estrogens and the osteocyte network play an integral part in maintaining bone equilibrium.

Bone microdamage accumulation has been implicated in osteoporotic fractures and increasing skeletal fragility. ⁽⁸⁾ Through the repair of microdamage, bone remodelling not only prevents the accumulation of old fragile bone but also results in the formation of new viable osteocytes. ⁽⁹⁾ Experimentally a strong association between microdamage, osteocyte apoptosis, and bone remodelling has been demonstrated. ⁽⁷⁾ This supports the hypothesis that osteocyte apoptosis provides a key part of the activation or signalling mechanisms by which osteoclasts target bone for remodelling. Bisphosphonates are a family of drugs which have proven successful in preventing bone loss and reducing fracture risk in osteoporosis. Etidronate has been shown to preserve bone mass in healthy postmenopausal women ⁽¹⁰⁻¹²⁾ and to increase bone mineral density (BMD) in the spine and hip. ^(13,14) Alendronate has also been found to substantially reduce the frequency of fractures among women with low bone mass and

existing vertebral fractures.⁽¹⁵⁾ Similarly, zoledronic acid increases BMD in postmenopausal women ⁽¹⁶⁾ and improves bone structure and mechanical strength in the long bones of ovariectomised rats. ⁽¹⁷⁾ Zoledronic acid also prevents loss of bone structure and mechanical strength in vertebral bone in ovariectomised rats. ⁽¹⁸⁾

Bisphosphonates work by inhibiting osteoclast bone resorption. ⁽¹⁹⁾ However, part of their success may also lie in their capacity to preserve the mechanosensitive osteocyte network. Bisphosphonates have been shown to have an anti-apoptotic affect on osteocytes both *in vivo* and *in vitro*,⁽²⁰⁾ along with causing the apoptosis of osteoclasts. ^(21,22) More recently risedronate has also been shown to suppress osteocyte apoptosis induced by fatigue loading of the ulna in rats. ⁽²³⁾ One possible problem with bisphosphonates suppressing bone remodelling is in relation to microdamage accumulation. As the removal of microdamage is an integral part of remodelling, preventing this process from occurring can result in the accumulation of microdamage. A number of studies have found that suppression of bone remodelling by bisphosphonates increases microdamage accumulation. ⁽²⁴⁻²⁷⁾

Aim

The aim of this study was to identify the effects of estrogen withdrawal following ovariectomy and a supra-pharmacological dose of zoledronic acid on the levels of osteocyte apoptosis and microdamage in an ovine model of osteoporosis.

Materials and Methods

Animal Model

Sixty-four skeletally mature (> 4years), mixed breed ewes were randomly assigned into an ovariectomy (OVX, n=32) or control group (n=32) on which no operative

procedure was carried out. Twelve months post-OVX, half of each group were sacrificed. A further eight months later (following 20 months of estrogen deficiency) four OVX animals were randomly selected to serve as a third group, OVX plus zoledronic acid (Zol; Novartis Pharma). Each animal received a 5mg dose of zoledronic acid in 100mls of saline infused over 30 minutes. This procedure was repeated for a further four weeks, giving each animal a 25mg dose. Following administration of the drug, the animals were returned to pasture. All remaining animals were sacrificed 31 months post-OVX, approximately one year following administration of zoledronic acid.

Apoptosis Staining

Following sacrifice a 3cm thick block of bone was removed from the distal end of the left metacarpal and fixed in 10% formalin. The samples were then decalcified by agitating in 0.5M ethylenediamine tetraacetic acid (EDTA), pH 7.2 at 4°C with a change of solution every 3 days. Once decalcified, samples were embedded in paraffin wax and cut to a thickness of 7µm using a microtome (Leica RM2255, Leica Microsystems, Germany) and placed on poly-L lysine coated slides. To detect apoptotic cells, the slides were stained using a DeadEnd Fluorometric terminal deoxynucleotidyl transferase-mediated dUTP- biotin nick end labeling (TUNEL) system (Promega Corporation, Madison, WI). The fluorescein-12-dUTP labelled deoxyribonucleic acid (DNA) was visualised directly by fluorescence microscopy using a standard fluorescein excitation and emission filter at 520+/- 20nm. Both a positive and a negative control were included in the staining process. For the positive control, DNase (RQ1 Dnase, MSC, Dublin, Ireland) was used to cleave DNA. The negative control was prepared using autoclaved water in place of the rTdT enzyme. A

mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei of all cells. Two sections from each bone sample were analysed.

Microdamage Detection

Adjacent to the block removed for apoptosis, a 3cm block of bone was removed from the distal end of the metacarpal. The sample was *en bloc* stained in basic fuchsin⁽²⁸⁾ before 100µm thick transverse sections were cut. Two slides from each sample were viewed using fluorescence microscopy and microcracks were identified using a previously described technique.⁽²⁸⁾

Statistical Analysis

Results are presented as mean \pm standard deviation. Results were tested for normality using the SPSS software package (SPSS Inc, Chicago, IL). ANOVA and the Mann-Whitney rank sum tests were used to determine statistical significance. A p value of ≤ 0.05 was considered to be significant unless otherwise stated.

Results

In this study there was no significant change in the osteocyte density following ovariectomy or zoledronic acid treatment (Table 1). After 12 months the control group had a value of $402 \pm 147 \text{mm}^{-2}$ while in the OVX group that value was $442 \pm 95 \text{mm}^{-2}$. At 31 months the density in the control group was $489 \pm 100 \text{mm}^{-2}$, in the OVX group the density was $550 \pm 98 \text{mm}^{-2}$ and in the Zol group it was $506 \pm 46 \text{mm}^{-2}$. However a significant increase in the level of osteocyte apoptosis was found in the OVX group relative to the control group at both time points (Figure 1). At 12 months approximately 1% of the osteocytes in the control group were undergoing apoptosis. Following ovariectomy this value rose to 10%. After 31 months there was no change

in the level of apoptosis in the control group, remaining at 1%. However at this later time point the level of apoptosis in the OVX group had increased to 15%. The results showed that there was no significant change in the level of apoptosis between the control groups over time. However, the levels of apoptosis were significantly higher at 31 months than at 12 months in the OVX group. Figure 1 also shows the anti-apoptotic effect that zoledronic acid had on osteocytes. Treatment with zoledronic acid resulted in a reduction in the level of osteocyte apoptosis, down to 3%.

No significant difference was found in the mean microcrack density between the control and OVX groups at either time point (Figure 3). In the 12 month group the mean crack density in the control group was $0.045 \pm 0.040 \text{ mm}^{-2}$ and in the OVX group it was $0.036 \pm 0.042 \text{ mm}^{-2}$. In the 31 month group, the mean microcrack density in the controls was $0.040 \pm 0.045 \text{ mm}^{-2}$ and $0.055 \pm 0.043 \text{ mm}^{-2}$ in OVX group. There was no significant change in mean crack density in the control group over time ($p=0.08$). There was no significant increase detected in mean crack density in the OVX group over time, ($p=0.07$). In the Zol group, the crack density of $0.225 \pm 0.043 \text{ mm}^{-2}$ was significantly greater ($p \leq 0.005$) than either the control or OVX groups.

In the 12 month group there was no significant difference in the mean microcrack length between the two groups (Figure 4). The mean crack length in the control group was $151 \pm 47 \mu\text{m}$ and in the OVX group it was $122 \pm 28 \mu\text{m}$. In the 31 month group again there was no significant difference in the mean crack length between the control ($148 \pm 35 \mu\text{m}$) and OVX ($130 \pm 29 \mu\text{m}$) groups. However, the average crack length was significantly less in the Zol treated animals ($84 \pm 25 \mu\text{m}$). There was no significant difference in mean crack length over time in either the control or OVX groups.

Discussion

This study found that estrogen withdrawal significantly increases the level of osteocyte apoptosis as early as 12 months. However, changes in microdamage accumulation are only becoming apparent after 31 months. Zoledronic acid treatment was shown to reduce the level of osteocyte apoptosis following estrogen withdrawal. Zoledronic acid treatment also significantly increased microdamage levels while significantly reducing the mean length of these cracks relative to both control and OVX groups.

Ovariectomy is often used as a model of post-menopausal osteoporosis. In sheep, 12 months of estrogen deficiency following ovariectomy leads to significant reductions in bone mineral density with changes seen as early as 6 months post OVX.⁽²⁹⁾ In the current study the time points were chosen to reflect changes previously reported. The initial 12 month group coincides with known BMD decreases. In order to maximise the changes seen following estrogen deficiency, the animals were left at pasture for a further eight months before zoledronic acid was administered (20 months post-OVX). As zoledronic acid is an annual treatment the animals were euthanized approximately 12 months later, providing the second time point of 31 months.

Sheep bone is primarily plexiform bone up until the age of 3-4 years⁽³⁰⁾ after which Haversian remodelled bone becomes more prevalent.⁽³¹⁾ At the starting point of this experiment our animals were 4+ years of age. Therefore, at the time points chosen (12 and 31 months post OVX) animals were 5+ years and approximately 7+ years of age. From other work carried out by our group, histological images from the metatarsal of the same animals show secondary osteons in the control group at 12

months, indicative of secondary remodelling occurring.⁽³²⁾ Another study by our group which examined the effects of bone turnover on crack behavior following fatigue testing found no significant difference in crack density between the control and OVX groups (after 12 months) in cracks <100um and between 100 and 300um long.⁽³³⁾ As most cracks which occur under normal physiological loading conditions (as was the case in the current study) fall within this range, we can assume that although some plexiform bone may remain this has little or no bearing on the current results and our ovine model is an accurate representation of the human condition.

The current prescribed dose of zoledronic acid for osteoporosis treatment is 5mg delivered as one dose annually.⁽³⁴⁾ As mature sheep have a similar weight and bone size to adults, 5mg annually can also be considered a clinical dose in sheep. However, patients receiving zoledronic acid for breast cancer treatment can receive a dose up to 270mg.⁽³⁵⁾ Bisphosphonates prevent bone turnover and as a result it is proposed that microdamage will accumulate following treatment with bisphosphonates. Therefore, the current study used a supra-pharmacological dose to reduce bone turnover and produce a marked microdamage accumulation in our small experimental group.

This study found no significant difference in the total number of osteocytes between the control and OVX groups. This is consistent with previous work which found that ovariectomy in sheep did not produce a significant change in osteocyte density.⁽³⁶⁾ In the current study, the level of osteocyte apoptosis was significantly greater in the OVX groups relative to the control groups. This increase in osteocyte apoptosis following ovariectomy indicates the importance of estrogens in maintaining osteocyte viability. In rats it has been shown that ovariectomy resulted in an increase of

osteocyte apoptosis from 2.3% in sham operated animals to 10% in ovariectomised animals. ⁽³⁷⁾ This is consistent with the changes in the current study where ovariectomy induced apoptosis from a level of approximately 1% in the control group to 10% in the ovariectomy group after 12 months, rising to 15% after 31 months. Zoledronic acid treatment produced an anti-apoptotic effect, reducing levels to approximately 3%.

While most studies on microdamage tend to examine the ribs or long bones such as the femur or tibia, in the current study the metacarpal was chosen for analysis. In sheep, unlike in humans, the metacarpal is a load bearing bone which is perpendicular to the ground when the animal is standing. Therefore, it is subject to continual fatigue loading, optimal conditions under which to produce microdamage. While this study found no significant difference in microdamage levels between the control and OVX groups, the values are consistent with previously reported data. The current study found crack values in the region 0.04 mm^{-2} and *in vivo* crack densities in the region of $0.012\text{-}0.074 \text{ mm}^{-2}$ have been reported in sheep radii. ⁽³⁸⁾ Similar values were reported in dogs with densities of between 0.042 and 0.073 mm^{-2} reported in the femur. ⁽³⁹⁾ In human metatarsals microcrack densities of $0.23 \pm 0.15 \text{ mm}^{-2}$ and $0.35 \pm 0.19 \text{ mm}^{-2}$ have been reported in cadaveric samples ⁽⁴⁰⁾ while in young males and females, crack densities in femoral compact bone of approximately $0.1\text{-}0.2 \text{ mm}^{-2}$ have been measured. ⁽⁴¹⁾ Therefore the values found in this study are consistent with both human and animal studies which determined *in vivo* crack densities.

In the current study it appears that prolonged estrogen deficiency may result in an increase in the mean microcrack density over time (between 12 and 31 month OVX

groups, $p=0.07$). In related studies carried out by our group using the same animals, the bone turnover was examined in the left metatarsal.^(32,42) These works found that in the ovariectomised animals there was a significant increase in bone turnover at 12 and 31 months. Therefore, it is hypothesised that the increased turnover is removing damage and may explain why no significant difference in crack density levels are seen between the control and OVX groups. However, turnover has slowed down in the 31 month OVX group relative to the 12 month group and there is a trend towards increased microcrack density between the 12 and 31 month OVX groups ($p=0.07$).

The current work also found an increase in crack density in those animals treated with zoledronic acid. Bisphosphonates have previously been reported to increase crack density and crack surface density at both clinical and supraclinical doses.^(24-26,43,44) Bisphosphonates prevent bone resorption, thereby older bone can accumulate which may result in an increase in damages levels as microcracks are not being removed. One reason why increased damage levels found following bisphosphonate therapy has not led to increased fragility following long term treatment might be partially explained by the current study. Although zoledronic acid treatment produced more cracks, the cracks that were formed were significantly shorter than those found in either the control or OVX groups. This might be explained by our understanding of microcrack growth and bone microstructure. The lamellar structure of osteons has been proposed as a mechanism to stop cracks produced by cyclic loading.⁽⁴⁵⁾ Subsequent to this the microstructure of cortical bone has been shown to have a significant influence on the propagation and initiation of microcracks.⁽⁴⁶⁾ In the ovariectomised group, the increased remodelling has resulted in the formation of extra secondary osteons. In a related study on the metatarsals of these animals, fatigue

loading *ex vivo* showed that cracks grew to significantly longer lengths in the control bone than they did in the ovariectomised bone.⁽³³⁾ In the current study the animals which received zoledronic acid had been ovariectomised 20 months prior to treatment. This provided an opportunity for the formation of a large number of osteons which might act as barriers to crack propagation and result in short cracks. Studies in our laboratory have also shown the existence of a microstructural barrier effect in bone.^(47,48) This concept describes how cracks not only slow down or stop at microstructural features, such as resorption cavities or osteons but that they also initiate easily in weaker areas, such as lamellar interfaces. It has previously been demonstrated that microdamage also initiates more readily in highly mineralised areas of bone.⁽⁴⁹⁾ As bisphosphonates increase bone mineralisation, the animals which were treated with zoledronic acid have more potential for the formation of microcracks. Therefore a supra-clinical dose of zoledronic acid following estrogen deficiency appears to encourage the formation of numerous small cracks and minimise the formation of larger, detrimental cracks.

This study has a number of limitations, one of which is that due to institutional ethical guidelines, a sham operated group was not used and rather a non-operated control was included. This was unlikely to have significantly impacted on this study, in particular limb loading, as those animals which underwent ovariectomy were subject to anaesthesia for a short period of time (<30mins) and once the anaesthesia had worn off, the animals were immediately mobile. The animals used in this study were also of mixed breed. There was no significant difference in bone mineral density or weight (data not shown here) between the breeds and therefore all animals were combined. However, it is possible that the variables examined in this study may be influenced by

breed and thus having a mixed population may affect the results. The total number of samples tested per group also differed due to the small sample size of the zoledronic acid treated group. While this is a limitation, the strong statistical significance seen would likely only be improved using larger sample sizes. The animals in this group also received a suprapharmalogical dose of zoledronic acid. As the aim of this study was to determine the effects of ovariectomy (increased bone turnover) and zoledronic acid treatment (reduced bone turnover) on microdamage accumulation and osteocyte apoptosis a high dose was necessary to achieve a near complete cessation of bone turnover. This study identified microdamage using fuchsin bulk staining and epifluorescence microscope which visualises the cracks in two-dimensions. Other studies have used alternative fluorochromes such as calcein which have a more specific binding than fuchsin.⁽⁵⁰⁻⁵³⁾ There has also been a trend towards examining microdamage in three-dimensions using confocal microscopy.^(47,50,54,55) Both of these techniques may help to eliminate any erroneous labelling of artefacts as microcracks. Nonetheless, basic fuchsin labelling remains the gold standard in the field.

Conclusion

Overall this study has demonstrated that estrogen withdrawal is accompanied by increased osteocyte apoptosis and that osteocyte viability can be preserved by zoledronic acid treatment. While zoledronic acid treatment was found to increase microdamage accumulation, the cracks produced are significantly shorter than in the control or ovariectomised groups.

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Table 1: Mean (\pm standard deviation) osteocyte density, % apoptotic osteocytes, microcrack density and microcrack length measured in sheep metacarpi following estrogen deficiency and zoledronic acid treatment.

	12 Months		31 Months		
	Control	OVX	Control	OVX	Zol
Osteocyte Density (mm⁻²)	402 \pm 147	442 \pm 95	489 \pm 100	550 \pm 98	506 \pm 46
% Apoptotic Osteocytes	0.8 \pm 0.5	9.7 \pm 3.0 ^a	0.4 \pm 1.4	14.7 \pm 4.6 ^{a,c}	3.3 \pm 5.6 ^b
Mean Crack Density (mm⁻²)	0.045 \pm 0.04	0.038 \pm 0.04	0.049 \pm 0.05	0.052 \pm 0.05	0.227 \pm 0.09 ^a
Mean Crack Length (μm⁻²)	151 \pm 48	126 \pm 27	140 \pm 35	132 \pm 29	84 \pm 33 ^a

Figure 1: TUNEL staining found a significant increase in the level of osteocyte apoptosis in the OVX group relative to control at both 12 and 31 months post-OVX (a, $p \leq 0.005$). The % apoptosis in the OVX group increased over time with a significant increase after 31 months relative to 12 months post-OVX (c, $p \leq 0.05$). Treatment with zoledronic acid significantly reduced the level of apoptosis (b, $p \leq 0.05$).

Figure 2: Fuchsin stained microcrack (white arrow) viewed using green incident light.

Figure 3: Mean microcrack density was significantly higher in the zoledronic acid treatment group than any other (a, $p \leq 0.005$).

Figure 4: The mean microcrack length was significantly lower following treatment with zoledronic acid than in any other group (a, $p < 0.05$).



